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Methylbromide: Carcinogenic Effects in the Rat Forestomach

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Methylbromide: Carcinogenic Effects in the Rat Forestomach. DANSE, L. H. J. C., VAN VELSEN, F. L., AND VANDER HEIJDEN, C. A. (1984). *Toxicol. Appl. Pharmacol.* 72, 262-271. Methylbromide (MB) administered by oral gavage as a solution in arachis oil was carcinogenic to rats in a 90-day experiment. In 13 of 20 animals of the highest dose group, 50 mg MB/kg body wt squamous cell carcinomas of the forestomach developed. All animals of this group showed a marked diffuse hyperplasia of the epithelium of the forestomach. A less pronounced hyperplasia was observed in high and lower incidence with respectively 10 and 2 mg MB/kg body wt. The lowest dose, 0.4 mg MB/kg body wt was without effects.

Methylbromide (MB) has found widespread pesticidal use as a soil fumigant in glass houses and for protection of stored food. In The Netherlands alone, approximately 2000 tons per year were used until recently for these purposes. Residues of MB are encountered in food due to illegal or injudicious use. Recently, it was found that MB had penetrated into drinking water through pipes underneath extensively fumigated areas of soil. Since humans might be exposed orally to MB, the subchronic oral toxicity of MB was investigated. Because of the reactive and volatile nature of the compound, it was decided to perform a gavage study with an oil solution instead of a more appropriate feeding or drinking water study.

In the literature, only limited data on oral experiments with MB are available. Dudley and Neal (1942) found the minimum single lethal dose for rabbits to be 60 to 65 mg/kg body weight. Miller and Haggard (1943) observed that rats died 5 to 7 hr following administration of 100 mg MB/kg body wt, dissolved in olive oil and given by gavage. We estimated an oral LD50 value for rats of 214 mg MB/kg body wt, dissolved in arachis oil. No other oral toxicity studies are known,

mainly because MB is a gas at temperatures above 4°C.

From an unpublished preliminary study, it was observed that daily gavage of 50 mg MB/kg body wt dissolved in arachis oil during 4 weeks induced epithelial hyperplasia, hyperkeratosis, and ulceration in the forestomach of rats. In the present 90-day study, attention was focused on effects on the forestomach.

METHODS

Monobromomethane (methylbromide, MB; BDH; purity > 98%) dissolved in arachis oil was administered by gavage 5 times a week during 13 weeks (gavage tube: length, 47 mm; e.d. 2 mm). The doses were 0, 0.4, 2, 10, or 50 mg/kg body wt. The administered volume was 10 ml/kg body wt. MB dissolved in arachis oil is stable for more than a month both at 4 and 20°C. Therefore, stock solutions were prepared monthly. MB in oil was analyzed by gas chromatography by a head-space method with an acetone-water (9:1, v:v) mixture. The measured concentrations deviated less than 10% from the intended ones.

Weanling Wistar rats Riv:TOX (M) weighing 40 to 60 g were littermate allocated to the control and the dose groups, 10 animals of either sex per group. The animals were kept 2 of similar sex per cage. The ambient temperature was $22 \pm 2^\circ\text{C}$; the relative humidity was $50 \pm 5\%$; and there was an 8-hr-light, 14-hr-dark schedule. Semisynthetic purified feed (SSP-Tox Standard from

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Trouw Ltd., Putten, The Netherlands) and tap water were available *ad libitum*.

Body weights were determined at the start of the experiment and weekly, thereafter. Feed consumption was determined three times per week throughout the study. The animals were handled daily and observed for clinical symptoms.

For hematological examinations, 1 week prior to termination of the study, a small blood sample (approx 1 ml) was taken from the retro orbital sinus under light ether anesthesia.

At the end of the experiment, the animals were anesthetized with ether and killed by bleeding from the abdominal aorta. To minimize diurnal variation, rats were killed at random between 9.00 AM and 2.00 PM. After gross examination, samples from 35 organs and tissues were fixed in 10% buffered Formalin. The stomach was opened along the major curve and stretched for fixation, after which two mediolongitudinal sections were made for microscopy. For the histopathological study, 5- μ m-thick Paraplast sections were cut from a selected number of organs such as stomach (all groups), liver, spleen, esophagus (control and 50 mg/kg group), and lungs (control, 10, and 50 mg/kg group). The sections were stained with hematoxylin and eosin (H&E). Sections of the spleen (control, 2-, 10-, and 50-mg/kg group) were stained according to Perl's method for detection of hemosiderin. Sections from the stomach (control, 0.4-, 2-, and 10-mg/kg group) and spleen (Perl's staining) were read under code to avoid reader bias.

Statistical analysis of data was performed with the two-sided Student's test. In case of inhomogeneity of variances, the number of degrees of freedom was corrected according to Welch.

RESULTS

General

During the study, the appearance and general behavior of the animals did not appear to be affected. One female of the 2-mg/kg group died after 8 days of treatment. At necropsy, oil was found in trachea and lungs indicating that the cause of death was improper intubation. One male of the 50-mg/kg group had to be killed in Week 10. The animal was cachectic. The results of the pathological examinations of this animal are included in Tables 3 and 4.

Body Weight Gain

From Fig. 1, it appears that from the start of the experiment the body weight gain of the

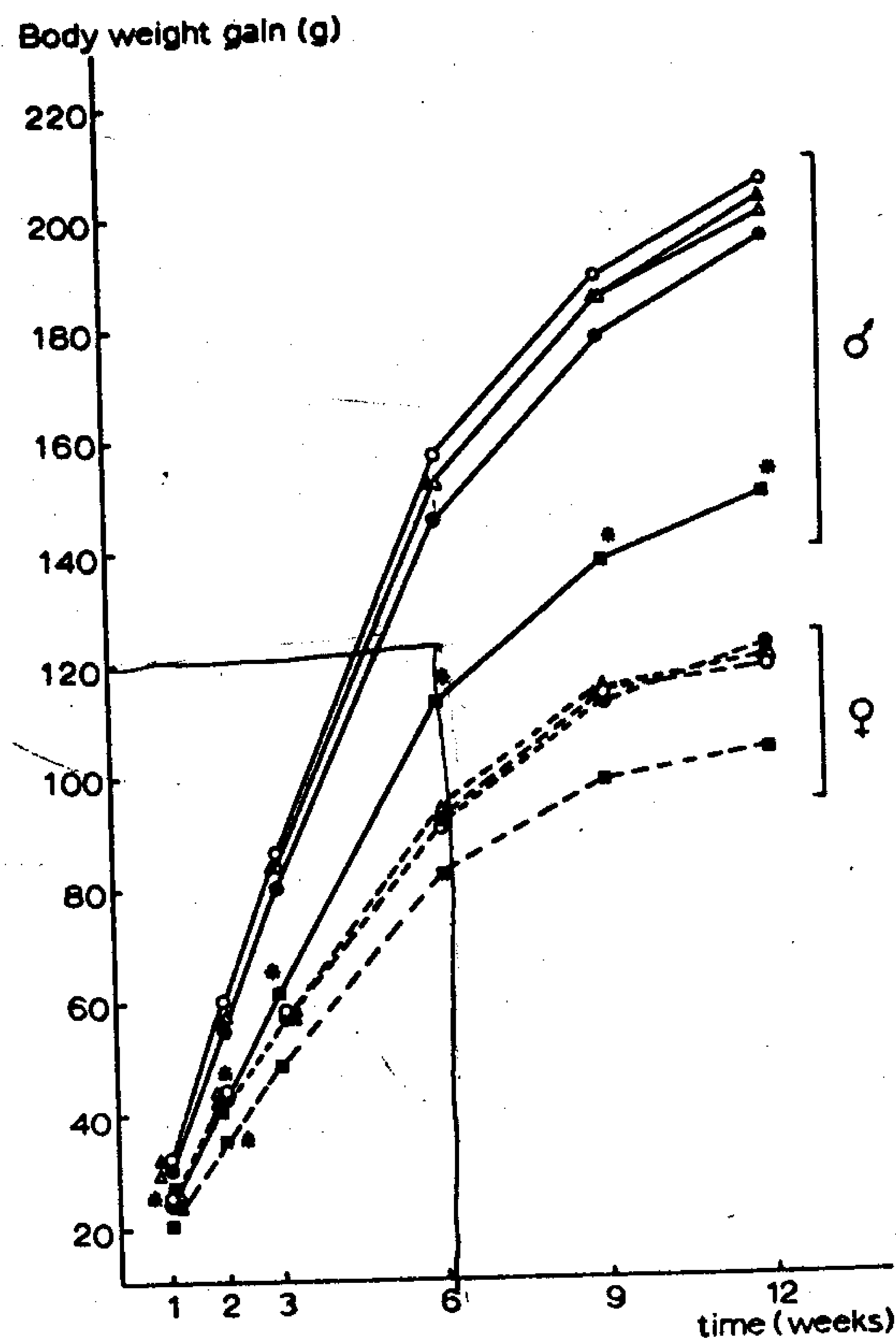


FIG. 1. Mean body weight gain of rats ($n = 10$) during a 90-day gavage study with methylbromide (MB) dissolved in arachis oil. (○) vehicle; (●) 0.4 mg MB/kg body wt; (Δ) 2 mg MB/kg body wt; (▲) 10 mg MB/kg body wt; (■) 50 mg MB/kg body wt; * $p < 0.001$.

males of the 50-mg/kg group was significantly less than that of the control animals. No statistical significance was observed for females.

Food Consumption

The mean food consumption of both males and females of the 50-mg/kg group was significantly lower than that of the control animals throughout the study (Table 1). In addition, at the end of the experiment, food consumption in the male animals of the 10- and 2-mg/kg groups was slightly but significantly lower.

Hematology

There was a slight anemia in the males of the 50-mg/kg group (Table 2): slightly lower

TABLE 1
FOOD CONSUMPTION OF RATS DURING A 90-DAY GAVAGE STUDY WITH METHYLBROMIDE
DISSOLVED IN ARACHIS OIL

Dose	Food consumption ^a in week				
	1	2	3	6	9
Females					
Vehicle	19	21	21	20	21
0.4 mg MB ^b /kg body wt	18	20	21	20	19
2 mg MB/kg body wt	18	20 (4) ^c	21 (4)	19 (4)	18 (4)
10 mg MB/kg body wt	20	22	22	22	21
50 mg MB/kg body wt	17*	19*	20	19	18*
Males					
Vehicle	22	25	28	30	30
0.4 mg MB/kg body wt	21	25	26	27	25
2 mg MB/kg body wt	22	25	27	28	26*
10 mg MB/kg body wt	21	24	26	27*	24**
50 mg MB/kg body wt	18***	20**	22*	24*	23*

^a Mean amount (g) of food eaten daily by 2 rats per cage. Number of cages is 5.

^b Methylbromide.

^c For the values in parentheses, $n \neq 5$.

* $0.01 \leq p < 0.05$.

** $0.001 \leq p < 0.01$.

*** $p < 0.001$.

RBC and Hb and slightly elevated MCV. Furthermore, a slight increase in WBC was observed in both males and females of the highest dose group. The neutrophilic granulocyte counts were increased twofold whereas lymphocyte counts were slightly higher. No significant differences were observed in the other experimental groups.

Gross Lesions

Several animals of the two higher dose groups showed proliferative alterations of the forestomach mucosa, characterized by hyperkeratosis and papilloma formation (Table 3). In addition, on group comparisons of the stretched forestomachs, it was observed that the total surface was decreased. All animals of the highest dose groups had developed an adhesive peritonitis which was associated in a number of animals with ulceration of the forestomach. An increasing incidence of focal hyperemia of the forestomach wall was noticed from the lowest dose group on.

Microscopic Lesions

Relevant microscopical lesions in the various organs are given in Table 4. In 13 of 20 animals of the highest dose group, the lesions in the forestomach were diagnosed as well-differentiated squamous cell carcinomas (Fig. 2) (Nagayo, 1973). In 2 animals a papilloma was observed. In general, the carcinomas showed only minimal exophytic growth. In 9 of the affected animals, the carcinoma showed minimal invasion (Fig. 3); in the remaining 4 animals, clear invasion through the muscularis mucosae (Fig. 4) was noticed. Generally, the mitotic index was increased. Keratinizing cells in the basal layer or isolated keratinizing cell nests in the submucosa were often seen (Fig. 5). In some animals the tumor was associated with ulceration and a proliferative inflammatory reaction in the underlying layers of the stomach wall. This reaction is considered to be partly a result of the developing tumor because the epithelial defects

TABLE 2

MEAN HEMATOLOGICAL PARAMETERS OF RATS AT THE END OF A 90-DAY GAVAGE STUDY WITH METHYLBROMIDE DISSOLVED IN ARACHIS OIL

		Females		Males	
		Vehicle	50 mg MB ^a /kg body wt	Vehicle	50 mg MB/kg body wt
No. of animals		10	10	10	9
RBC ^b		8.34	8.45	8.84	8.24*
Hb ^c		9.3	9.3	9.7	9.4
PCV ^d		0.43	0.43	0.44	0.43
MCV ^e		51.4	50.3	49.9	52.4*
MCH ^f		1121	1103	1101	1137
MCHC ^g		21.6	21.8	21.9	21.5
Thrombocytes (10 ⁹ liter ⁻¹)		923	928	857	922
WBC ^h (10 ⁶ liter ⁻¹)		9740	12540**	12840	14044
Differentiation					
Eosinophilic	Granulocytes	78	127	133	146
Basophilic		15	20	15	0
Neutrophilic		1000	1978	1441	2337*
Lymphocytes		8288	10064*	10896	11253
Monocytes		360	351	355	308

^a Methylbromide^b RBC, red blood cell concentration (10¹² liter⁻¹).^c Hb, hemoglobin concentration (mmol liter⁻¹).^d PCV, packed cell volume.^e MCV, mean corpuscular volume (fl).^f MCH, mean corpuscular hemoglobin (amol).^g MCHC, mean corpuscular hemoglobin concentration (mmol liter⁻¹).^h WBC, white blood cell concentration (10⁶ liter⁻¹).* 0.01 ≤ *p* < 0.05.** 0.001 ≤ *p* < 0.01.

might also provoke an inflammatory response. Sometimes the inflammatory process extended through the stomach wall and gave rise to local peritonitis with adhesion of the stomach to adjacent organs. In addition to these local processes, the forestomach squamous epithelium showed a strong diffuse hyperplasia and hyperkeratosis. Hyperplasia was characterized by increase and rearrangement of atypical basal cells, increased mitosis, and a marked downward out-growth of the basal layer (Fig. 6). To a lesser degree this hyperplasia was also noticed in the 2- and 10-mg/kg groups.

Lesions of the esophageal wall, e.g., hemorrhages and myodystrophy, were only seen in a few animals of the highest dose group. In one animal, which died before the end of

the experiment, extensive ulceration and an inflammatory reaction in the esophageal wall were noticed. Hyperplastic lesions as observed in the forestomach were not detected.

Microscopical examination of the lungs of animals of the highest dose group revealed no metastases of the squamous cell carcinomas. A slightly increased incidence of focal interstitial pneumonia and slight atelectasis, which were seen in the two higher dose groups, might be due to inhalation of small amounts of MB-containing oil during gastric intubation. In the liver no metastases of the carcinomas or other treatment related lesions were found. Decreased hemosiderosis and increased hematopoiesis in the spleen of males of the highest dose group were noticed.

TABLE 3

INCIDENCE OF MACROSCOPICAL LESIONS IN TISSUES AND ORGANS OF RATS AT THE END OF A 90-DAY GAVAGE STUDY WITH METHYLBROMIDE DISSOLVED IN ARACHIS OIL

	mg MB/kg body wt									
	0		0.4		2		10		50	
	No. of animals: 10									
	Sex: ♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Forestomach										
Hyperkeratosis							3	3	2	5
Decreased surface							3	2	8	9
Papilloma							1		1	
Ulcer									5	2
Focal hyperemia				1	4	1	8	10	1	
Fundic stomach										
Focal hemorrhage										1
Adhesive peritonitis									10	10
Esophagus										
Focal hemorrhage										1
Spleen										
Atrophy										1

DISCUSSION

This study shows that MB administered daily for 90 days by gavage as a solution in arachis oil induces tumors in the forestomach of the rat. The tumors were diagnosed as well-differentiated squamous cell carcinomas. Both the high incidence of these tumors and the very short induction time were unexpected. In aged rats of the strain used, the spontaneous incidence of this tumor is very low (Kroes *et al.*, 1981).

The malignancy of the tumors was based on their intensive and infiltrative growth, increased mitotic index, and the presence of keratin-forming cells in the basal layer and submucosa. The diffuse hyperplasia of forestomach epithelium, which was seen in a dose-related incidence in the three higher dose groups, also showed a marked downward outgrowth with slight atypia of basal cells suggesting a preneoplastic stage.

Since MB is a very reactive compound, the localization of the neoplastic lesions was

closely connected with the site of application. The lesions occurred in the forestomach exclusively, although this part of the stomach is protected by a keratinized epithelium.

We considered the reduction of the body weight gain, the decreased food consumption, the granulocytosis, the decreased hemosiderosis, and increased hematopoiesis in the spleen together with the mild anemia to be secondary to the lesions in the forestomach of the highest dose group.

Mutagenicity studies with MB have been performed by Voogd *et al.* (1982). MB appeared to have mutagenic properties in four of five tests. In a test with *Klebsiella pneumoniae* at concentrations of 4.75×10^3 mg/m³ air and higher, in an Ames-test with *Salmonella typhimurium* TA 100 at concentrations of 1.9×10^3 mg/m³ air and higher, in a gen mutation test with L5178 Y mice lymphoma cells at concentrations of 0.3 mg/liter suspension and higher, and in a test for sex linked recessive-lethal mutations with *Drosophila melanogaster* at 375 mg/m³ air after

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TABLE 4

INCIDENCE OF HISTOLOGICAL AND HISTOCHEMICAL LESIONS IN RATS AT THE END OF A 90-DAY GAVAGE STUDY WITH METHYLBROMIDE DISSOLVED IN ARACHIS OIL

	mg MB/kg body wt									
	0		0.4		2		10		50	
	No. of animals: 10 Sex: ♀	10 ♂	10 ♀	10 ♂	10 ♀	10 ♂	10 ♀	10 ♂	10 ♀	10 ♂
Forestomach examined	10	10	10	10	10	10	10	10	10	10
Diffuse hyperplasia										
Slight	1		1		1	2	9	6	2	
Strong									8	10
Papilloma										2
Carcinoma									6	7
Ulcer									1	2
Inflammatory reaction submucosa									8	6
Adhesive peritonitis									2	4
Esophagus examined	10	10							10	10
Hemorrhage submucosa									2	
Focal myodystrophy tun. muscularis									3	
Ulcer										1
Lung examined	10	10					10	10	10	10
Focal interstitial pneumonia	1						3	2	2	
Slight atelectasis		1							5	4
Spleen examined	10	10			10		10	10	10	10
Hemosiderosis (Perls' staining)										
Slight	4	2			3		2	5	6	
Moderate	6	7			7		8	5	3	
Extramedullary hematopoiesis	1	1			n.e. ^a		n.e.	1	3	
Adhesive peritonitis					n.e.		n.e.	2	2	

^a n.e., not examined.

5 expositions of 6 hr each, and at 200 mg/m³ air after 15 expositions of 6 hr each, this mutagenic potential was established. The reported concentrations of the *Drosophila* tests were the highest tested without lethal effect on the flies. A DNA-repair test in primary rat hepatocytes did not show results of mutagenic potential at concentrations from 10 to 30 mg/liter medium.

A finding of particular significance to the carcinogenic effects of MB is the rapid induction of diffuse hyperplasia of the epithelium of the forestomach. In a preliminary experiment, hyperplasia associated with epithelial damage was observed after 4 weeks of treatment. Therefore, the diffuse hyperplasia appears to occur as a reaction to the direct cytotoxic effects of MB. We regard MB as a

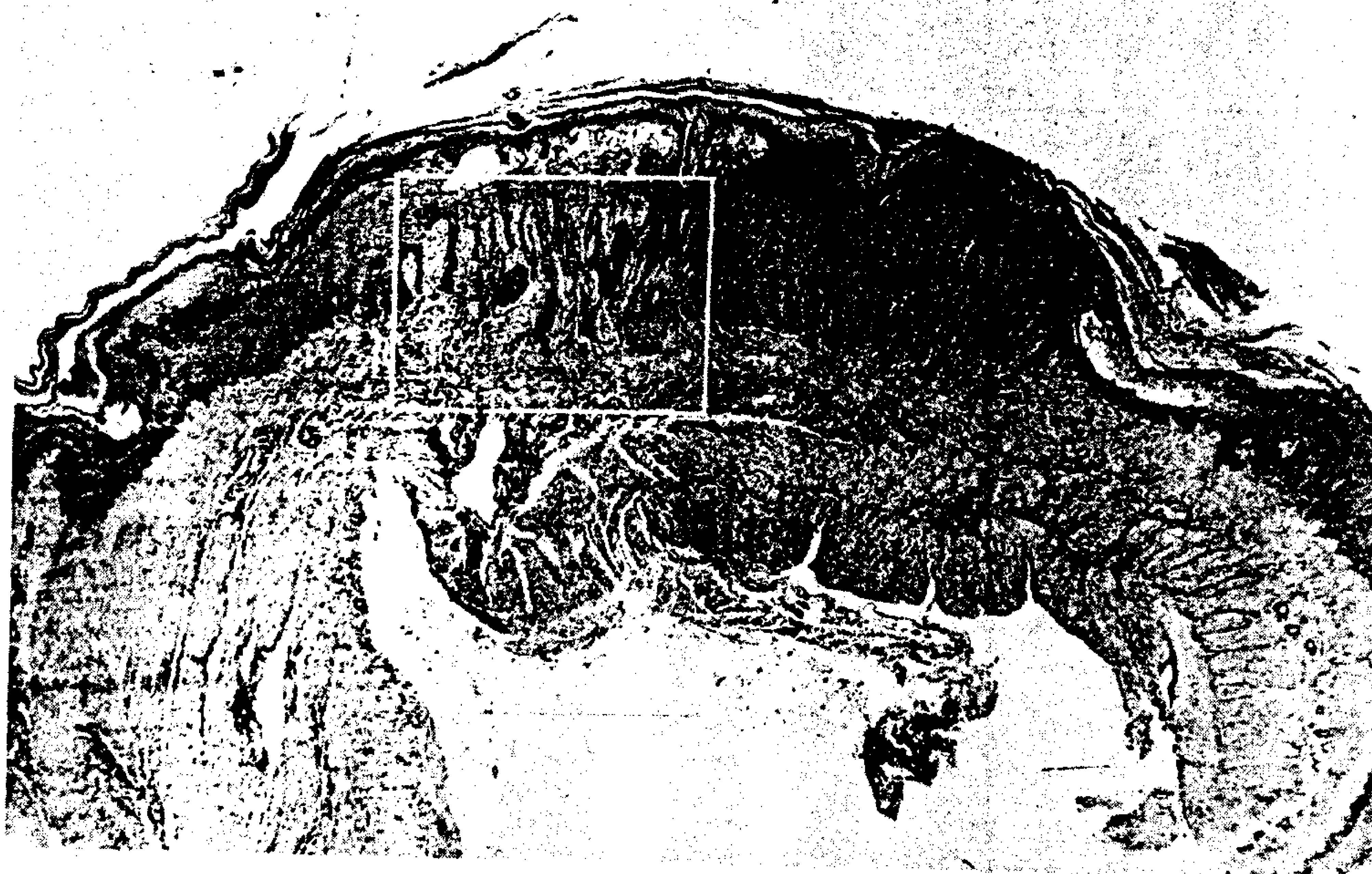


FIG. 2. Well-differentiated squamous cell carcinoma in forestomach of rat of the highest dose group showing only local invasion in the submucosa. H&E $\times 24$.

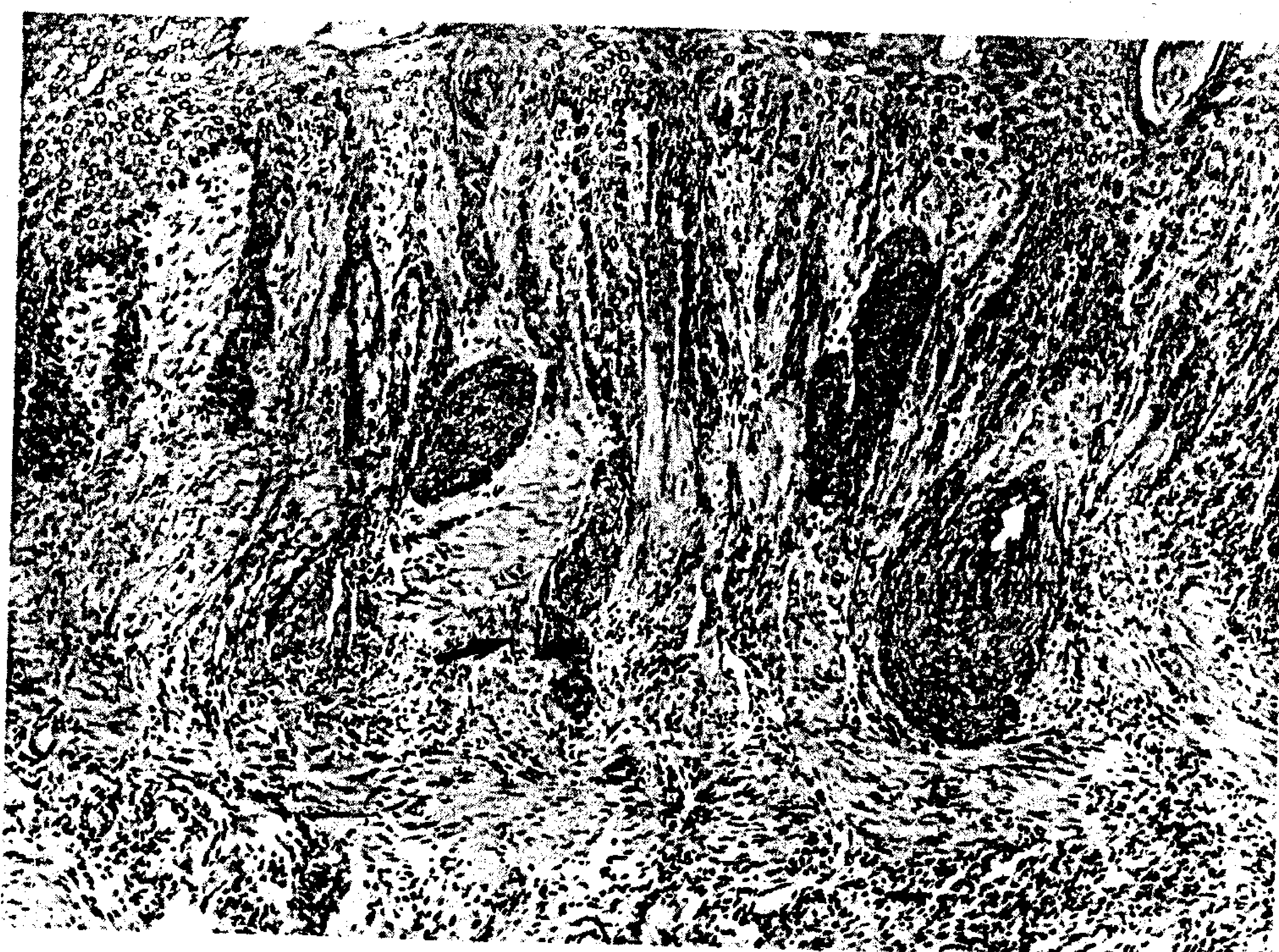


FIG. 3. Detail from tumor of Fig. 2 showing strong local infiltrative growth of basal cells (arrow). H&E $\times 93$.



FIG. 4. Carcinoma in forestomach of rat of the highest dose group with invasion of the basal layer through the muscularis mucosae. H&E $\times 93$.



FIG. 5. Carcinoma in forestomach of rat of the highest dose group with strong infiltrative growth of basal layer and isolated keratinizing basal cells (arrow) in the submucosa. H&E $\times 230$.

highest dose group

ls (arrow). H&E

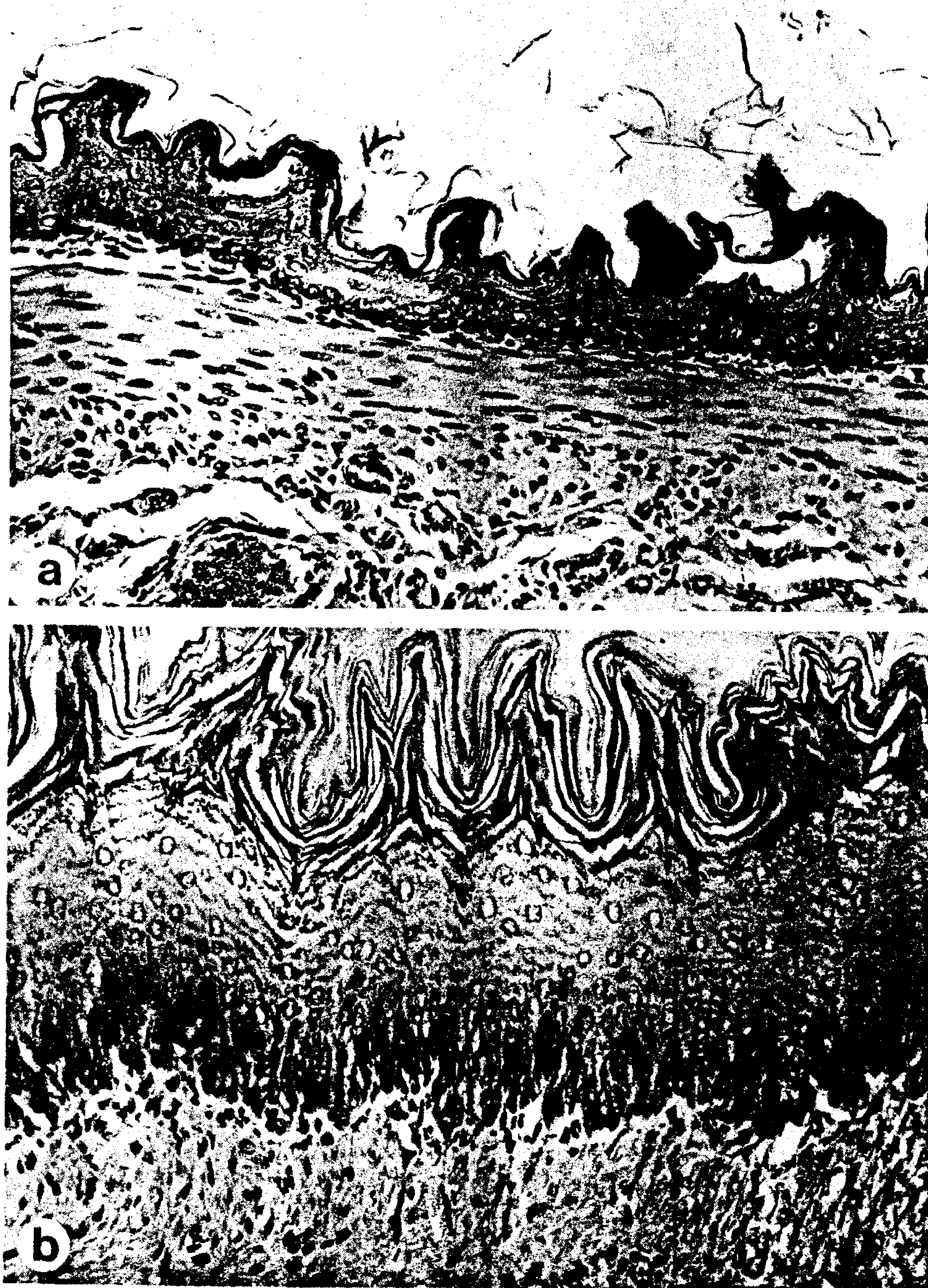


FIG. 6. Forestomach of rat. H&E $\times 230$. (a) Control animal with normal keratinizing epithelium. (b) Animal from the highest dose group showing epithelial hyperplasia and hyperkeratosis with marked downward out-growth of atypical basal cells.

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It is likely that the induced hyperplasia by MB played a major role in the genesis of the squamous cell carcinomas in the highest dose group. In spite of the potential initiating activity of MB, it might be speculated that at low exposure levels without apparent promotor activity (i.e., hyperplasia) the risk for a carcinogenic response is very small.

The initial purpose of this study was to establish a no toxic effect level of MB focusing on the effects on the forestomach. However, because of the unexpected carcinogenic response in this 90-day rat study it is not possible to estimate a no effect level based on a threshold dose. A low dose extrapolation to establish human risk by a mathematical model (e.g., linear nonthreshold or multistage model) is also not justified because of the interfering effects of cytotoxicity. To make a proper quantification of risk for humans exposed orally to very small amounts of MB, further studies are necessary to elucidate the significance of cytotoxicity in the genesis of the squamous cell carcinomas.

The major pathway of exposure to MB is by air, in particular for humans living in areas with a high frequency of soil fumigation practice. The presented experimental data do not permit quantification of the risk for humans

exposed by inhalation. The results of chronic inhalation studies are needed for this purpose. A 2-year rat study is in progress.

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